

Expression of Chinese hamster cAMP-dependent protein kinase in *Escherichia coli* results in growth inhibition of bacterial cells: A model system for the rapid screening of mutant type I regulatory subunits

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ABSTRACT The regulatory and catalytic subunits of cAMP-dependent protein kinase (PKA) were coexpressed within the same bacterial cell using a polycistronic bacterial T7 expression vector encoding Chinese hamster cDNAs for the type I regulatory (RI) and catalytic α ($C\alpha$) subunits of PKA. Basal expression of active RI/ $C\alpha$ holoenzyme in the BL21(DE3) strain of *Escherichia coli* caused severe growth inhibition resulting in extremely small colony size. Several lines of evidence demonstrate that this growth inhibition requires active PKA subunits and cAMP: (i) this phenotype is dependent on cAMP since it is not seen in a strain lacking adenylyl cyclase activity, but the growth rate of these transformants is slower when exogenous cAMP is added; (ii) normal growth occurs when wild-type RI cDNA is replaced by a mutant RI cDNA encoding a RI protein with reduced cAMP binding; and (iii) the growth-inhibited phenotype of the transformed BL21(DE3) cells requires soluble, active $C\alpha$ protein. Holoenzyme expressed in bacteria is activated by cAMP, which stimulates phosphorylation of an endogenous 50-kDa protein that is missing in four host mutants selected for normal growth after transformation with PKA holoenzyme. A mutant RI cDNA library was generated by PCR random mutagenesis and screened by polycistronic expression in BL21(DE3) cells. The RI cDNA sequence from one revertant has base-pair substitutions creating two amino acid substitutions within the cAMP binding sites. The coexpression of the RI/ $C\alpha$ subunits in BL21(DE3) bacterial cells provides a system for rapidly selecting mutations in the RI subunits of PKA.

The intracellular second messenger cAMP mediates cellular responses via activation of the cAMP-dependent protein kinase (PKA) (reviewed in refs. 1 and 2). The type I PKA is a heterotetramer composed of two regulatory type I (RI) subunits (49-kDa proteins) and two catalytic (C) subunits (40-kDa proteins) (1, 2). Different isoforms of both subunits have been isolated, characterized, and cloned from mouse, bovine, human, and, recently, hamster sources (refs. 3–9; reviewed in ref. 2). Both the RI and C subunits have been expressed independently in *Escherichia coli* (6, 10, 11). The C subunit isoforms have differing solubilities when expressed in the *E. coli* BL21(DE3) Studier expression system; $C\alpha$ is partially soluble and $C\beta$ is predominantly insoluble (6, 11). The soluble RI protein and soluble $C\alpha$ protein expressed in bacteria can be reconstituted *in vitro* to generate cAMP-activatable holoenzyme (6, 11).

Although several mutants affecting the cAMP binding sites of PKA have been described (2), these sites have not been completely defined, nor has the mechanism by which cAMP causes dissociation of RI and C to activate PKA been determined. In the past, we have used cAMP mediated

growth inhibition of Chinese hamster ovary (CHO) cells to select a limited number of PKA mutants for this analysis. We and others have used a Chinese hamster full-length cDNA of the wild-type RI protein, generated site-directed mutants, expressed these mutant RI cDNAs in the BL21(DE3) expression system, and biochemically characterized the mutation in holoenzyme reconstitution studies (6). However, these mutants are not easily screened for function in mammalian expression systems, and many such mutants are not phenotypically dominant over endogenous wild-type RI subunits. Perhaps more than one amino acid substitution is required for the clear ablation or inhibition of RI subunit function in these mammalian experimental systems (12).

To enable rapid screening of mutants of RI and C which block PKA activation by cAMP, we have coexpressed the RI and $C\alpha$ subunits of the Chinese hamster type I PKA within the same bacterial cell and have found that activation of PKA by endogenous cAMP inhibits bacterial growth. This growth-inhibition phenotype can be used to screen for mutants in the PKA system which are not activated by cAMP.

METHODS

Materials. [γ - 32 P]ATP was from Amersham, histone type IIA was from Sigma, and the large fragment of DNA polymerase I (Klenow fragment) was obtained from GIBCO/BRL. The BL21(DE3), BL21(DE3)/pLysS, and BL21(DE3)/pLysE strains were a gift from W. Studier (State University of New York at Stony Brook) (13, 14) and the BL21(DE3)/ Δ cya strain was prepared by P1 transduction as described (6). The pVEX-11 vector was a generous gift of V. Chaudhary (National Institutes of Health, Bethesda, MD) (15). BL21(DE3) cells were grown in LB broth, BL21(DE3)/pLysS and BL21(DE3)/pLysE were grown in LB broth with chloramphenicol (15 μ g/ml), and all strains transformed with the pVEX constructs were grown in LB broth supplemented with ampicillin (50 μ g/ml) (16).

Preparation of the Polycistronic Expression Vector. A 1.1-kb *Xba*I–*Hind*III fragment excised from pT7C α (a gift from R. Maurer, University of Iowa) (7) was blunt-ended by incubation with the large fragment of DNA polymerase I (Klenow fragment) (17). This $C\alpha$ fragment, containing a ribosome binding site (Shine–Dalgarno sequence) followed by the full-length Chinese hamster $C\alpha$ cDNA, was blunt-end cloned (17) into the blunt-ended *Hind*III site of pVEXRI (6). An *Asp*718 site was generated in the vector by replacing the *Hind*III–*Nde*I fragment with the oligodeoxynucleotide se-

Abbreviations: PKA, cAMP-dependent protein kinase; IPTG, isopropyl β -D-thiogalactopyranoside.

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quence 5'-AAAGCTTTGTTTAACTTTAAGAAGGAGG-TACCT-3'. Thus the final construct contains specific cloning sites for RI (*Nde* I, *Hind*III) and $C\alpha$ (*Asp*718, *Bam*HI) cDNA molecules. The resultant vector (Fig. 1) was sequenced (18) to verify the restriction enzyme sites and Shine-Dalgarno sequences (15).

Preparation of a Mutant Chinese Hamster $C\alpha$ cDNA (KG72) by PCR. Two oligodeoxynucleotides of 30 bases were generated which contained GGG (Gly-72) substituted for AAG (Lys-72): 5'-AACCACATATGCCATGGGGATCTTGGACAAACAG-3' and its complement. Two other 30-mers were generated to anneal to the 5' and 3' ends of the $C\alpha$ cDNA to create an *Asp*718 and *Bam*HI site at the 5' and 3' ends, respectively; the sequences are 5'-CATATGCATATGGCAACGCCGCCGCCGCC-3' and 5'-GGATCCGGATCCCTAAACTCAGTAAACTC-3'. One PCR amplified a 234-bp fragment as defined by the 5' cDNA primer with the 3' primer containing the desired base changes, and a second PCR amplified a 790-bp fragment as defined by the 5' primer containing the base-pair changes and the 3' cDNA primer. The full-length $C\alpha$ cDNA was amplified by using the 234-bp and 790-bp amplified fragments as the templates and the 5' and 3' cDNA primers. The resultant 1055-bp fragment was cloned into the pVEX polycistronic bacterial expression vector (see above).

In Vitro Phosphorylation by the RI/ $C\alpha$ Holoenzyme. Logarithmic phase cultures derived from individual colonies transformed with the RI/ $C\alpha$ vector were diluted 1:100 (final volume, 50 ml), grown to midlogarithmic phase, incubated in 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) for 1 hr, harvested, and suspended in 0.5 ml of TEM buffer [10 mM Tris-HCl, pH 7.4/1 mM EDTA, with phenylmethylsulfonyl fluoride (15 mg/ml) and aprotinin (Sigma, 1% by volume)]. The suspended cells were frozen and thawed three times on dry ice, sonicated, and centrifuged at $12,000 \times g$ for 20 min at 4°C (6). The supernatant was dialyzed overnight in 4 liters of TEM buffer with one change of buffer. Protein concentration was determined with bovine serum albumin as standard (19). Bacterial extract (7.5 μ g of protein) was incubated in 50 mM Mops, pH 7.0/10 mM $MgCl_2$, in the absence or presence of 1 μ M cAMP, 1 mM ATP (25 μ Ci of [γ - 32 P]ATP; 1 Ci = 37 GBq) at room temperature for 30 min, and analyzed by SDS/PAGE (stacking gel, 3.5%; resolving gel, 10% poly-

acrylamide) followed by autoradiography on Kodak X-Omat AR film (6).

Isolation of Bacterial Host Mutants. pVEXRI/ $C\alpha$ -transformed BL21(DE3) cells were mutagenized by nitrosoguanidine (16), and 324 colonies of wild-type size were picked from LB agar plates containing ampicillin (50 μ g/ml). Twenty-seven host mutants from the original 324 colonies which retained wild-type colony size were tested for growth on MacConkey sorbitol plates with ampicillin (50 μ g/ml). Eighteen mutants were eliminated (white colonies on the MacConkey sorbitol plates) as adenyl cyclase or cAMP receptor protein mutants (20). Plasmids were isolated from the remaining 9 mutants and tested for ability to induce small colonies after transformation of wild-type BL21(DE3) cells; plasmid copy number and integrity of the plasmid (as determined by *Sca* I digestion) were also tested. Four host mutants (PKr-1, PKr-2, PKr-3, PKr-4) with normal plasmids and one control transformant (PKwt-1) with an intact plasmid which maintained the small phenotype after the mutagenesis protocol were further studied.

Preparation of the Random RI Mutant cDNA Library. The *Nde* I-*Hind*III fragment of RI cDNA was amplified by 30 cycles of PCR using a 5' primer containing an *Nde* I site and a 3' primer containing a *Hind*III site (see above). The conditions for generation of adenine substitutions were as follows: 61 mM $MgCl_2$; 2 mM $MnCl_2$; 1 mM dGTP, dCTP, and dTTP; 0.25 mM dATP, 0.5 unit of *Taq* polymerase, and 10 ng of DNA in a final volume of 100 μ l (21). The RI mutant amplified cDNA was digested with *Nde* I and *Hind*III, ligated into the polycistronic expression vector, and used to transform BL21(DE3) cells. Approximately 10% of the plated colonies resulted in the revertant phenotype (wild-type colony size). Thirty-two revertant colonies were selected and purified. The plasmids from these mutants were tested for maintenance of the revertant phenotype in wild-type cells, plasmid copy number, and plasmid integrity. One plasmid, pVEXRI/ $C\alpha$ 3.9, satisfied the above criteria and the RI cDNA was sequenced with the DuPont 2000 automated sequencer (22).

RESULTS

Expression of Chinese Hamster PKA Inhibits Growth of *E. coli*. Expression of the Chinese hamster RI and $C\alpha$ subunits of PKA in *E. coli* results in partially soluble proteins (6). Coexpression of RI and $C\alpha$ subunits in the same bacterial cell might allow RI/ $C\alpha$ interaction resulting in soluble, functional holoenzyme. The pVEX-11 bacterial expression vector was engineered to contain both RI and $C\alpha$ cDNAs (Fig. 1). Low level "leaky" basal coexpression of RI and $C\alpha$ produced a dramatic reduction in colony size when compared with vector, RI, $C\alpha$, $C\beta$, or RI/ $C\beta$ expression (Fig. 2). Two control constructs—pVEXWR261RI/ $C\alpha$, encoding a mutant RI subunit [WR261 (6)] and pVEXRI/KG72 $C\alpha$, encoding a $C\alpha$ (KG72) with a mutation in the putative ATP binding site—did not result in growth inhibition of BL21(DE3) cells. The microcolony phenotype was also not seen after transformation of pVEXRI/ $C\alpha$ into *E. coli* BL21(DE3)/pLysS and BL21(DE3)/pLysE (data not shown), in which the basal activity of the T7 polymerase-dependent promoter is suppressed (14).

The effect of coexpression of RI and $C\alpha$ subunits on the growth of BL21(DE3) cells was studied (Fig. 3A). The host cells and most of the vector-transformed cells had similar growth curves. The cells transformed with $C\alpha$ showed a lag period before logarithmic growth. The soluble $C\alpha$ protein in this transformant was mostly insoluble by 6 hr of growth (Fig. 3A *Inset*); this corresponds to the initiation of logarithmic growth in these cells and demonstrates the requirement of active, soluble $C\alpha$ protein for the growth-inhibitory pheno-

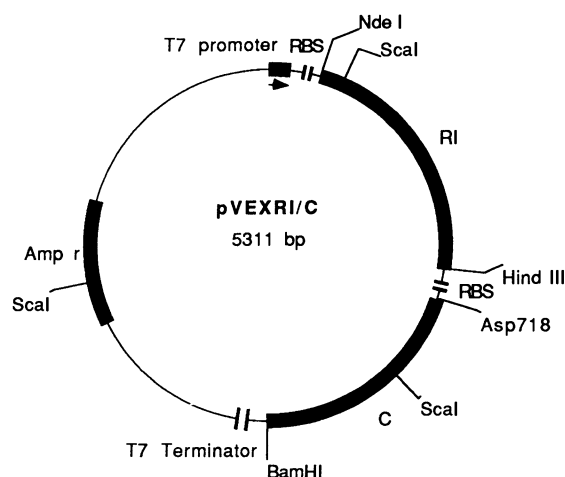


FIG. 1. Schematic diagram of the polycistronic bacterial expression vector for the hamster RI and $C\alpha$ or $C\beta$ subunits of PKA. RI cDNA was inserted between the *Nde* I and *Hind*III sites, and $C\alpha$ or $C\beta$ cDNA was inserted between the *Asp*718 and *Bam*HI sites. *Sca* I digestion of this construct was used to determine if any major deletions of the plasmid had occurred during the transformation or by growth in the host BL21(DE3) strain. RBS, ribosome binding site. Amp r, ampicillin-resistance gene.

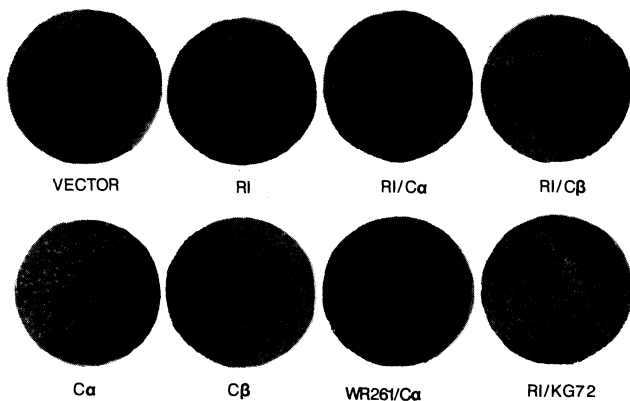


FIG. 2. Growth inhibition of BL21(DE3) bacteria by "leaky" coexpression of the RI and $C\alpha$ subunits of PKA. pVEXRI (6), pT7C α (see *Methods*), pVEXC β , and the pVEXRI/C constructs (as listed) were used to transform *E. coli* BL21(DE3). The cells were plated on LB plates with ampicillin (50 μ g/ml) and incubated overnight at 37°C without IPTG.

type. The cells transformed with RI/C α had a greater lag than the cells transformed with C α alone. Unlike the C α transformant, where C α became insoluble over time, the C α subunit when coexpressed with the RI subunit was soluble during the entire 6-hr growth experiment (data not shown). After the initial lag for either RI/C α or C α alone, all the transformants grew with similar growth rates. The overnight cultures from these transformants had a similar optical density (data not shown). When IPTG was added to the culture to induce expression of RI and C α , the optical density of RI/C α transformants remained at ≤ 0.1 for at least 6 hr (data not shown).

Growth Inhibition of BL21(DE3) Cells by PKA Is Dependent on cAMP. The growth-inhibitory effects of the coexpression of RI and C α required endogenous cAMP. When a strain of BL21(DE3) lacking adenyl cyclase, BL21(DE3)/ Δ cya (6), was transformed with the pVEXRI/C α construct, these transformants (Fig. 3B, \square) grew at a rate similar to the parental strain transformed with vector alone. Growth of the BL21(DE3)/ Δ cya RI/C α transformants was reduced in the presence of cAMP (\blacksquare) similar to the BL21(DE3) RI/C α transformant (Fig. 3B, \circ , and A, \blacksquare). In the same experiment (Fig. 3B), the growth-inhibitory effect of the RI/C α basal coexpression in BL21(DE3) cells (\circ) was greatly enhanced by addition of cAMP (\bullet).

Evidence for Soluble and Active RI/C α Holoenzyme. Coexpression of RI and C α in BL21(DE3) and BL21(DE3)/ Δ cya resulted in soluble proteins of equivalent amount (Fig. 4 and data not shown). Equal amounts of RI and C α were also present as soluble proteins in similar quantities in the absence of IPTG (Fig. 4). Coexpression of a mutant RI cDNA [WR261 (6)] with C α resulted in soluble RI and C α proteins (data not shown). RI/C β or RI/KG72 coexpression resulted in the expression of soluble RI protein and insoluble C protein (data not shown).

To test whether RI/C α coexpression resulted in cAMP-activatable holoenzyme, cell-free lysates were DEAE-Sephacel-purified and analyzed for *in vitro* kinase phosphorylation of histone type II A. The holoenzyme prepared from RI/C α - and WR261/C α -transformed BL21(DE3) cells resulted in cAMP-activatable kinase activity (Table 1). The values for cAMP activation of the holoenzyme generated by the RI/C α coexpression were similar to those found by reconstituting bacterial expressed Chinese hamster RI and C α *in vitro* (6) for both wild-type and mutant RI holoenzymes.

Phosphorylation of Specific Endogenous Bacterial Substrates by PKA. A reasonable hypothesis for the phenotype of RI/C α expression within BL21(DE3) bacteria is that endog-

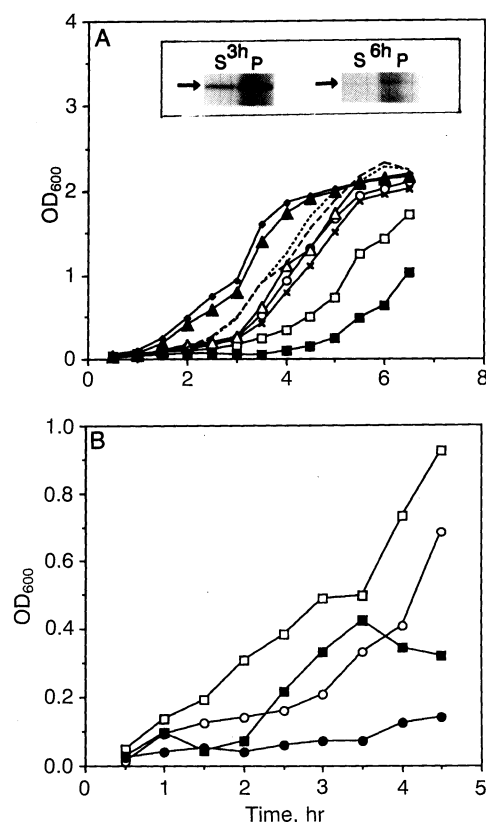


FIG. 3. Growth inhibition of BL21(DE3) cells by RI/C α holoenzyme is dependent on soluble C α and cAMP. Transformed colonies were selected on LB plates with ampicillin (50 μ g/ml), and a 1:100 dilution of an overnight culture was incubated in broth at 37°C. At the indicated time points, 1-ml aliquots were removed and OD₆₀₀ was determined. Plotted values are the mean of duplicate samples; data are representative of a typical experiment ($n = 3$). (A) \bullet , BL21(DE3); \times , vector; \circ , RI; \square , C α ; \triangle , C β ; \blacksquare , RI/C α ; \blacktriangle , RI/C β ; \cdots , WR261/C α ; and $- - -$, RI/KG72. (B) pVEXRI/C α transformants: \circ , BL21(DE3); \bullet , BL21(DE3) plus 1 mM cAMP; \square , BL21(DE3)/ Δ cya; \blacksquare , BL21(DE3)/ Δ cya plus 1 mM cAMP. (Inset) Ten milliliters of C α transformed BL21(DE3) cells from the experiment shown in A was used to prepare supernatant (S) and pellet (P) fractions (6). Protein (2 μ g per lane) was loaded for SDS/PAGE (stacking gel, 3.5%; resolving gel, 10% polyacrylamide) followed by Western blot analysis of the C α subunit using rabbit anti-C antiserum 4448 (6). Arrows indicate C α detected with ¹²⁵I-protein A by autoradiography for 36 hr.

enous cAMP causes the dissociation of the holoenzyme and the free C α subunit phosphorylates an important *E. coli* substrate(s) which affects growth. Phosphorylation of an endogenous 50-kDa *E. coli* substrate occurred only when wild-type holoenzyme was activated by cAMP (Fig. 5A). Soluble C α alone phosphorylated a 50-kDa protein and additional proteins when compared with the active holoenzyme or endogenous phosphorylated proteins. Phosphorylation of endogenous proteins of 18, 32, 55, 65, and 80 kDa was seen in all lanes.

Revertant Bacterial Host Mutants Resistant to Growth Inhibition Lack Phosphorylation of Specific Substrates by PKA. RI/C α -transformed BL21(DE3) cells were mutagenized with nitrosoguanidine, and cells resistant to the PKA holoenzyme-mediated growth-inhibitory phenotype were selected. Holoenzymes from four host mutants (PKr-1, PKr-2, PKr-3, PKr-4) and a control (PKwt-1) were DEAE-Sephacel-purified and tested for *in vitro* cAMP-activated kinase activity. All five holoenzymes had cAMP-induced phosphorylation of histone protein with an EC₅₀ of cAMP activation similar to that of wild-type holoenzyme (Table 1). After these

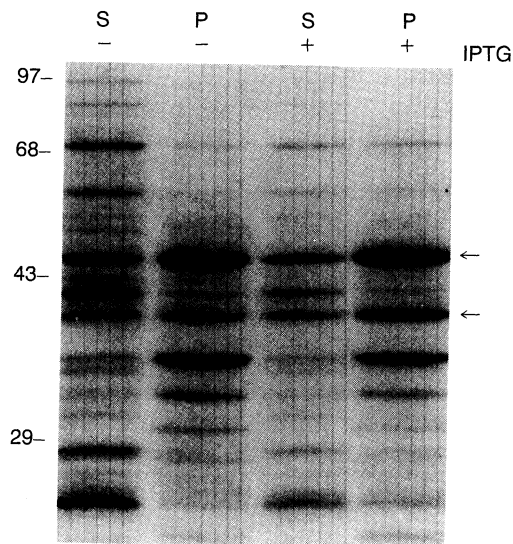


FIG. 4. Coexpression of the RI/C α subunits in BL21(DE3) cells results in soluble holoenzyme. pVEXRI/C α -transformed BL21(DE3) cells in midlogarithmic phase were either harvested (–, absence of IPTG) or incubated with 0.5 mM IPTG for 1 hr (+). Cell lysate supernatant (S) and pellet (P) fractions were prepared and 8 μ g of protein was electrophoresed and stained with Coomassie blue. Arrows indicate the position of the RI (49 kDa) and C α (40 kDa) proteins. Molecular size (kDa) markers are at left.

four host mutants were cured of the RI/C α bacterial expression vector and retransformed with fresh RI/C α expression vector, the colony size remained wild-type. Thus, these data provide evidence that the mutation in these mutants is in the host genome and not in the RI or C α cDNAs. *In vitro* phosphorylation showed a loss of the 50-kDa protein in all four mutants (Fig. 5B). A decrease in phosphorylation of proteins of \approx 18 kDa and a loss of a 70-kDa protein band were also seen in three of the mutants (PKr-2, PKr-3, and PKr-4).

Use of RI/C α Expression in BL21(DE3) Cells to Isolate a Mutant of RI. This system for coexpression of the RI and C α subunits has the potential of providing a rapid method for the screening of functional mutants of the RI subunit. To test this method, we prepared a mutant RI cDNA library by PCR. The mutant RI cDNA library in the pVEXRI/C α expression

Table 1. Holoenzyme activity of RI/C α constructs expressed in BL21(DE3) wild-type bacteria and host mutant RI/C α strains

BL21(DE3) cells	Kinase activity, % control		cAMP activation (EC ₅₀ , nM)
	– cAMP	+ cAMP (10 μM)	
Transformants			
Vector	1 ± 1	1 ± 1	—
RI/Cα	2 ± 1	97 ± 3	60 ± 17
WR262/Cα	2 ± 1	99 ± 4	163 ± 23
Host mutants			
PKr-1	5.2 ± 1.4	91.2 ± 1.0	63.0 ± 8.7
PKr-2	1.1 ± 0.6	96.1 ± 1.1	68.6 ± 5.1
PKr-3	4.2 ± 0.8	98.2 ± 1.3	64.3 ± 2.3
PKr-4	3.4 ± 1.0	97.1 ± 1.6	71.0 ± 9.5
PKwt-1	2.7 ± 0.9	98.6 ± 1.8	76.7 ± 6.5

BL21(DE3) transformants or host mutant cell cultures were grown to midlogarithmic phase, RI/C α protein expression was induced by incubation for 1 hr with 0.5 mM IPTG, cells were harvested, and cell-free lysates were prepared (6). PKA holoenzyme was partially purified by DEAE-Sephacel chromatography and was tested for kinase activity in the absence or presence of various concentrations of cAMP (6), and the concentration of half-maximal activation (EC₅₀) was calculated. —, Holoenzyme activity which was not different from the sample without substrate.

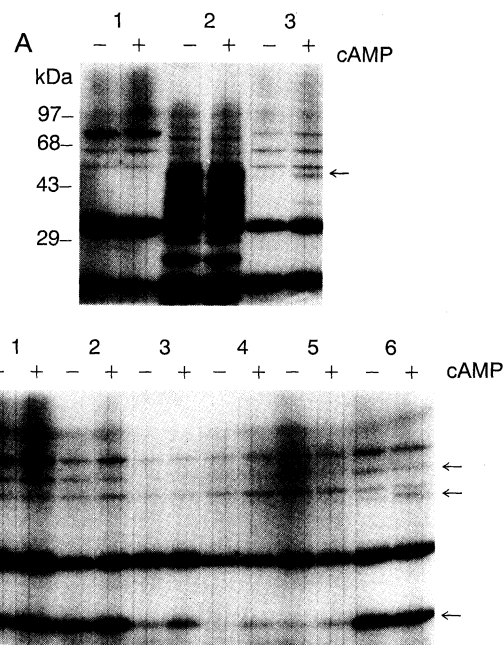


FIG. 5. cAMP activates recombinant bacterial PKA to specifically phosphorylate a 50-kDa protein that is absent in the host cell mutants. (A) BL21(DE3) cells (lanes 1) and pVEXC α (lanes 2) and pVEXRI/C α (lanes 3) transformants were grown to midlogarithmic phase and induced with IPTG. Cell-free lysates dialyzed overnight to remove endogenous cAMP were incubated in the absence (–) or presence (+) of 1 μ M cAMP and [γ -³²P]ATP in a Mops buffer. The arrow shows a protein whose phosphorylation is increased by cAMP. (B) Host mutant cell (lanes 1); lines PKr-1, PKr-2, PKr-3, and PKr-4 with revertant phenotype (wild-type size) (lanes 2–5, respectively); and PKwt-1, a transformant with the microcolony phenotype (lanes 6), were grown and induced with IPTG and dialyzed lysates were treated with cAMP as in A. Arrows indicate the 70-, 50-, and 18-kDa phosphorylated host substrates.

vector was then screened by coexpression with C α in the BL21(DE3) cells. Colonies of wild-type size were analyzed. Fifty percent (16 of 32) of the plasmids gave transformed BL21(DE3) colonies of wild-type size and did not exhibit major deletions of either RI or C α cDNA. One of these RI cDNAs contained two different base-pair substitutions representing Thr²⁰⁹ \rightarrow Ala and Glu²⁹¹ \rightarrow Gly, nonconservative amino acid changes well within the defined cAMP-binding region of RI (2).

DISCUSSION

Coexpression of the RI and C α subunits from Chinese hamster PKA inhibits growth of *E. coli* BL21(DE3). This reduction in the growth rate of the bacteria can be attributed to holoenzyme formation and subsequent activation by endogenous cAMP. Our data show a dependence of the growth-inhibitory phenotype on active holoenzyme and expression driven by T7 RNA polymerase, as well as a requirement for active PKA subunits and cAMP. The requirement for a phosphorylation event mediating growth inhibition is also shown by our ability to isolate bacterial host mutants lacking specific substrates for PKA-mediated phosphorylation. Host mutations altering specific target serine or threonine residues or changing accessibility of C α to protein substrates could produce this phenotype. This heterologous system can be used to select for mutations within the RI protein.

When the Chinese hamster C α subunit is expressed alone, the growth-inhibitory phenotype is not observed. Our data suggest that C α expressed alone is rendered insoluble and inactive with time. When complexed with RI, its solubility

appears to be preserved to allow activation of functional α by cAMP. In addition, soluble α expressed by itself may be subject to degradation by bacterial proteases.

During the isolation of the host mutants, 18 of 28 mutants were found to be sorbitol-negative on MacConkey sorbitol plates, suggesting that they are deficient in adenylyl cyclase or cAMP receptor protein activity. We expected to isolate adenylyl cyclase mutants since endogenous cAMP is needed for activation of the RI/ α holoenzyme. We have not determined whether any of these mutants lack cAMP receptor protein activity.

Expression of proteins from cDNAs using the BL21(DE3) expression system has been used to overproduce a variety of single proteins from many different sources (23–34). Coexpression of the D1 and D12 open reading frames of the vaccinia virus mRNA-capping enzyme within the same bacterial cell has been achieved (35). Generally, overexpression of a cDNA by the bacteriophage T7 RNA polymerase in BL21(DE3) does not result in a phenotype noticeably different from the wild-type. However, BL21(DE3) cell toxicity occurs after induction of expression of the type I DNA topoisomerase from vaccinia virus (33, 34) or the *pol* gene of human immunodeficiency virus (36). As in the system we describe, this toxicity could presumably be used to select for mutants in the heterologous cDNA.

Our use of mutagenesis by PCR allows the creation of double or triple mutants of the RI subunit, and preliminary data suggest that these multiple mutations may also generate tighter cAMP-resistance phenotypes (R.D.F., M.E.G., and M.M.G., unpublished data; ref. 12). This polycistronic bacterial expression system could be used to rapidly screen for multiple mutations of the RI protein, and these RI mutant proteins could be used in transfection studies to systematically study cAMP-mediated growth inhibition in mammalian cells.

Further, by selecting a large number of transformed colonies with both growth-inhibited and revertant phenotypes, it should be possible to create a catalog of mutations in the RI subunit which either affect function or have no effect on function. Such mutations can be used to map functional and nonfunctional domains of the RI protein. Such a catalog of mutations of RI will enhance our efforts to probe the structure–function relationships of PKA in mammalian cells.

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